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Age-Associated Oxidative Damage to the p62 Promoter: Implications for Alzheimer's Disease

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Abstract

Absence of the p62 gene in mouse brain leads to biochemical and cognitive deficits that resemble Alzheimer's disease (AD). In this context, the objective of this study was to examine the relationship between age-induced oxidative damage to the p62 promoter and AD. Increased 8-OHdG staining, a marker of oxidative stress, was observed in brain sections from mice deficient in the p62 gene compared to control. Treatment of MEF cells deficient in p62 with H₂O₂ resulted in decreased cell survival and an absence of Nrf2 nuclear translocation. The mouse p62 promoter exhibited elevated oxidative damage with increasing age and the degree of p62 promoter damage was also age-correlated in human brain samples. In human subjects, the expression of p62 was decreased in AD brain relative to age-matched controls, and likewise decreased p62 expression correlated with oxidative damage to the promoter. Treatment of HEK cells with H₂O₂ resulted in decreased p62 expression concomitant with increased promoter damage. Consistent with these findings, a transgenic AD mouse model also exhibited increased p62 promoter damage and reduced p62 levels in brain. Altogether, our results reveal that oxidative damage to the p62 promoter correlates with decreased expression of p62 and may contribute to age-associated neurodegenerative disease such as AD and others.

Keywords

DNA Oxidative damage; aging; Alzheimer's disease; p62 promoter

Introduction

Oxidative stress is the result of imbalance between productions of reactive oxygen species (ROS) and a biological system's ability to detoxify the reactive intermediates or repair the oxidative damage. Low levels of ROS can act as signaling molecules in various intracellular processes [1]. However, when the ROS level overwhelms the antioxidant and repair system, cells might be damaged by these molecules. Progressive and irreversible accumulation of oxidative damage contributes to the aging process, and is thus implicated in development of several age-associated diseases, such as neurodegenerative diseases [2]. Oxidative damage to DNA is particularly harmful since it may cause mutations that can be inherited by the next generation, leading to genome instability. Among the five nucleobases, guanine is the most susceptible to oxidation because of its high electron density [3].

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The predominant DNA oxidative adduct is 8-hydroxydeoxyguanosine (8-OHdG), which serves as a common biomarker of DNA oxidative damage [3,4]. Most 8-OHdG lesions are repaired by a base excision repair (BER) pathway. It has been reported that when the ability of the repair system declines, cumulative oxidative DNA damage in the mitochondria and nuclear genomes of neurons may play a critical role in brain aging and neurodegenerative disorders such as AD, Parkinson's disease and Amyotrophic Lateral Sclerosis [5,6, and 7].

Relationships have been established among increased DNA damage, defective DNA repair, aging, and age-associated neurodegenerative disease [8,9, and 10]. However, the exact mechanism by which oxidative DNA damage might lead to neurodegeneration or neuronal cell death still remains obscure. Human p62 is oxidatively-induced in human and mouse cell lines [11], and the p62 protein has been localized to aggresomes of various neurodegenerative diseases [12]. This gene was first identified by Park et al., as the ligand for the p56 *lck* [13], and referred to as Sequestosome 1/SQSTM1. In humans, variants of p62/SQSTM1 have been linked to Paget's Disease of Bone [14]. In mouse, the gene is related to A170/STAP (Signal Transduction Adapter Protein) [15]; whereas in rat, the gene is referred to as ZIP, the zeta interacting partner of the atypical protein kinase C [16]. The p62 protein contains several interaction motifs that endow the protein with scaffolding abilities [17]. At its C-terminal tail p62 possesses an ubiquitin associated domain that interacts with K63 polyubiquitin chains and the N-terminus possesses a PB1 domain. A ZZ- finger recruits the atypical PKC and other proteins, whereas the TRAF6 binding site (TBS) recruits the E3 ubiquitin ligase TRAF6. Functionally, p62 serves to connect signaling pathways associated with two post-translational modifications. An absence of p62 leads to the loss of aggresomes and neuronal cell death [18]. Moreover, p62 has been reported to activate the antioxidant response element (ARE) and protect cells from oxidative stress [19]. We reported that p62 deficiency in mice results in an AD-like phenotype [20]. These mice display age-dependent neurofibrillary tangles (NFTs), memory deficits, loss of synaptic plasticity, and accumulation of polyubiquitin.

Age is one of the main risk factors for AD [21]. Findings from an extensive study on transcription profiling of brain from aged-humans reveals that there exist a set of genes whose promoters are selectively damaged in an age-dependent fashion resulting in reduced expression [22]. The majority of these genes play a role in synaptic plasticity, vesicular transport and mitochondrial function, similar to the reported functional role for p62 [17]. The objective of this study was: 1) to determine if p62 promoter was damaged in an age-dependent manner in humans and mice; 2) to examine p62 expression in human brain from late-stage AD individuals; and, 3) to examine the relationship between p62 promoter damage and p62 expression. Altogether, these findings reveal that reduced p62 may be an additional risk factor for AD and other neurodegenerative diseases.

Materials and Methods

GenBank Information

Human p62: Sequestosome 1 (SQSTM1), Gene bank #: BC019111.1 [13]. Mouse p62: A170/STAP, Gene bank #: BC006019.1 [15]. Rat p62: ZIP, Gene bank #: BC061575 [16].

Human brain samples and cell lines

Post mortem samples of frontal cortex derived from normal human adult without neurological disease were provided by Dr. Steven Carroll at the University of Alabama Birmingham, Department of Pathology, Birmingham, AL. AD and age-matched control (normal human brain) samples (frontal cortex) were obtained from Emory University Alzheimer's Disease Research Center, Emory University, Atlanta, GA. AD cases met CERAD and NIA-Reagan Institute criteria for the neuropathologic diagnosis of AD [23,24]. Additional control samples

were obtained from the Harvard Brain Tissue Resource Center, McClean Hospital, Boston, MA. The control samples used in this study had no clinical history of neurological disease and were neuropathologically normal (Table 1). WT and p62^{-/-} Mouse Embryo Fibroblast (MEF) cells were obtained from Dr. Jorge Moscat, University of Cincinnati, Cincinnati, OH. A triple transgenic mouse model of AD (3xTg-AD) was provided by Dr. Frank Laferla, UCLA, Los Angeles, CA. Knock-out mice (p62^{-/-}) were generated as described previously [25]. All animals employed in this study were handled according to the Auburn University Institutional Animal Care and Use Committee, which abides by National Institutes of Health guidelines.

DNA isolation

Total DNA was isolated as described previously [22]. Genomic DNA was isolated from brain tissues by DNeasy Tissue Kit (Qiagen, Valencia, CA) with the following modifications to minimize *ex vivo* oxidation artifacts. All buffers were purged with nitrogen and supplemented with 50 μ M phenyl-tert-butyl nitron (PBN) (Sigma, St. Louis, MO). The high temperature incubation was replaced by 4 hours incubation at 37°C. Following elution with ddH₂O, purified DNA was stored at -80°C.

Oxidative DNA damage assay

The DNA damage assay was developed by Lu, et al. [22]. Quantitative real time PCR was employed to determine the intact DNA level of specific amplicons within both the human and mouse p62 promoter. Primers designed for each amplicon are shown in Table 2. In brief, the formamidopyrimidine glycosylase (fpg) (New England Biolabs, Ipswich, MA) cleavage reaction was performed by incubating 250 ng of total genomic DNA with 8 units of fpg and 100 μ g/ml of BSA in a total volume of 50 μ l at 37°C for 12 hours, followed by incubation at 60°C for 10 min. to inactivate fpg. An aliquot of the reaction mixture was used for quantitative PCR assay. Real time quantitative PCR was carried out on an ABI 7500 Real Time PCR system (Applied Biosystems, Foster City, CA) using Power SYBR Green PCR Master mix. All reactions were performed in a 25 μ l mixture containing 1X SYBR Green master mix, 0.2 μ M primer mix (forward and reverse), and template DNA for QPCR, respectively. A standard curve derived from 5-fold serial dilutions of genomic DNA was used to determine the absolute concentrations of intact DNA in the template. Oxidative damage was calculated as: (intact DNA in non-treated aliquot - intact DNA in fpg-treated aliquot)/(intact DNA in non-treated aliquot). Negative controls (absence of template for RT-PCR) were used to monitor nonspecific amplification. PCR products were verified by melting curves. Fluorescence was converted into DNA concentration using a standard curve. All DNA samples were analyzed three independent times.

8-OHdG immunohistochemistry

Clone N45.1 (JaICA, Tokyo, Japan), which specifically recognizes 8-OHdG was used for immunostaining [26,27]. After deparaffinization with Xylene, WT and p62^{-/-} brain sections (5 microns) were hydrated through graded ethanol. Endogenous peroxidase activity in the tissue was eliminated by incubation with 3% H₂O₂ in methanol for 15 min. The sections were treated with 20 μ g/ml proteinase K for 45 min. at 37°C and nonspecific binding sites were blocked by adding the mouse Ig blocking reagent in the MOM kit (Vector Lab, Burlingame, CA) for 4 hours. The sections were incubated with 7.5 μ g/ml anti-8-OHdG monoclonal antibody, N45.1 in MOM diluent overnight at 4°C, followed by addition of biotinylated anti-mouse IgG reagent. No primary antibody control was also performed. Next, ABC reagent was used to enhance the signal and immunostaining was developed by DAB for 3–5 min. Quantification of 8-OHdG was conducted employing the Gel and Graph Digital software (Silk Scientific Corporation, Orem, Utah). Five different regions were taken from WT sections with

corresponding regions examined from p62^{-/-} sections to quantify the amount of 8-OHdG per section.

Western blotting

Brain tissue was homogenized in 1 ml/gm of ice-cold 1 M sucrose in 0.1 M MES, 1 mM EDTA, 0.5 mM MgSO₄, pH 7, and centrifuged at 50,000 X g for 20 minutes at 4°C. Cytoplasmic fraction was collected from WT and p62^{-/-} MEF cells using Nuclear Extract Kit (Active Motif, Carlsbad, CA). Protein concentration was determined by Bradford assay (Bio-Rad Laboratories, Hercules, CA), using bovine serum albumin (BSA) as a standard. The lysate was subjected to SDS-PAGE in 10 % acrylamide gels. Samples were transferred from the gel to a nitrocellulose membrane. The blot was blocked with 7% milk in TBS-Tween (20 mM Tris, 8g/L NaCl, 0.1% Tween 20, pH 7.5) and incubated with primary antibody followed by secondary antibody. The blot was then processed with ECL reagent (Amersham Pharmacia Biotech, Pittsburgh, PA) for two min. and exposed to ECL film. Gel and Graph Digital software (Silk Scientific Corporation, Orem, Utah) was used to scan and quantify the signal.

Cell Viability Assay

WT and p62^{-/-} MEF cells were grown in Dulbecco's modified Eagle's medium (Gibco, Carlsbad, CA) supplemented with 10% fetal calf serum at 37°C in a 5% CO₂ humidified incubator. WT and p62^{-/-} MEF cells were seed in 96-well plate at 10,000 cells per well. After 48 h, the cells were treated with various doses of H₂O₂ for 18 h [19]. Each dose was tested in duplicate and the experiment replicated four independent times. Thereafter, cell viability was measured using CellTiter-Glo (Promega, Madison, WI).

Statistical analyses

Possible differences among group means and statistical relationships between the level of p62 expression and p62 promoter damage were analyzed using t-tests, ANOVAs and correlation analyses (SAS v9.1, SAS Institute Inc., Cary, NC, U.S.A.). For significant differences, global alpha was set at 0.05. Adjustments for multiple comparisons were derived using step down Sidak procedures (t-tests) or Tukey's studentized range tests (ANOVAs).

Results

Absence of p62 results in oxidative stress

The predominant DNA oxidative adduct, 8-OHdG, which is induced by ROS, serves as the biomarker for oxidative DNA damage [3,4]. The degree of oxidative damage was examined by analyzing matched brain sections from WT and p62^{-/-} mice stained with 8-OHdG at the same time. Since 6-month-old p62^{-/-} mice exhibit an AD phenotype [15], the levels of 8-OHdG in WT and p62^{-/-} brain sections were examined by immunostaining with a monoclonal antibody specific for 8-OHdG (N45.1) at 2-, 6-, and 12-months- of age [27]. Immunoreactivity for 8-OHdG was present in both cortical and hippocampal neurons, although hippocampal neurons in the CA1 to CA3 region of p62^{-/-} brain were more intensely stained (Fig. 1A). Absence of primary antibody did not yield any immunoreactivity. Both WT and p62^{-/-} mice displayed elevated levels of 8-OHdG with increasing ages and sections from p62^{-/-} brain possessed more intensive 8-OHdG reactive neurons, which was more apparent by 12 mos of age. Quantitative evaluation of 8-OHdG intensity was made by scanning the same unit area from each stained section of WT and p62^{-/-} mouse brain (Fig. 1B). These findings confirmed that both WT and p62^{-/-} mice displayed significant age-dependent elevated levels of 8-OHdG. Moreover, 8-OHdG levels in p62^{-/-} mouse brain sections were significantly higher than those in age-matched WT mouse brain sections. The 12- month-old p62^{-/-} mouse brain sections

possessed a significant increase in DNA oxidative damage compared to sections from either 2- or 6-month old brain, revealing that loss of p62 enhanced oxidative stress in brain.

What is the consequence in this loss of p62? P62 activates the antioxidant response element and confers resistance to toxicity induced by oxidative stress [19]. In order to investigate whether p62 has the ability to protect cells from oxidative insult, WT and p62^{-/-} MEF cells were treated with H₂O₂ for 18 h and cell viability was measured. Deficiency of p62 significantly decreased cell survival in response to H₂O₂ treatment, particularly at higher doses (Fig. 2A). The Nrf2 transcription factor regulates expression of the antioxidant response element by translocation to the nucleus. In mice deficient in p62, Nrf2 nuclear translocation was low [28], furthermore, decreased nuclear Nrf2 levels have been observed in neurons from AD brain [29]. Treatment of WT MEF cells with H₂O₂ resulted in decreased cytoplasmic Nrf2 without any alteration in tubulin levels. By comparison, Nrf2 failed to leave the cytoplasmic fraction in p62 KO cells treated with H₂O₂ (Fig. 2B), revealing that p62 regulates the activity of Nrf2. Collectively, these results suggest that p62 protects cells from oxidative stress through a Nrf2-dependent pathway [19,28].

P62 promoter is oxidatively modified in an age-dependent manner

Since p62 expression is regulated at the transcriptional level [18] and the p62 promoter contains a CpG island [30], which is sensitive to oxidative modification [22], studies were undertaken to examine if the p62 promoter is subject to age-dependent oxidative damage. To test for DNA damage, an amplicon was chosen within the mouse p62 promoter and three amplicons were designed within the human p62 promoter (Table 2; Fig. 3A). These amplicons were selected based upon their degree of GC richness (>60%) and the position of putative transcription factor binding sites [30]. A recently developed DNA damage assay [22] was validated with these amplicons and used to examine the degree of oxidative damage to the mouse p62 promoter in DNA samples isolated from mice with increasing age (Fig. 3B). Oxidative damage to the mouse p62 promoter increased significantly in 12-month-old brains compared to either the 2, or 6-month-old brains, but there was no significant difference between 2, and 6-month-old samples (Fig. 3B). This result was consistent with an increase in 8-OHdG levels with age observed by immunostaining (Fig. 1A, B). Parallel studies were undertaken to examine whether oxidative damage to the human p62 promoter was also age-related. Samples selected for this analysis were obtained from postmortem brain of individuals with no known history of neurological disease (Table 1). Sites further from the transcription initiation site are more difficult to repair than those which are closer [31]. Therefore, amplicon 3, which is furthest from the transcription initiation site (Fig. 3A), was selected for correlation analysis to examine if any relationship existed between the degree of oxidative damage exhibited by this amplicon and the age of the individual (Fig. 3C). A significant positive correlation was observed ($r = 0.589$, $p < 0.05$). Likewise, damage to amplicons 1 and 2 were also significantly correlated with age (A1: $r = 0.842$, $p < 0.05$; A2: $r = 0.934$, $p < 0.01$). Collectively, these findings reveal that the p62 promoter is subject to age-related oxidative damage in both mice and humans.

Oxidative damage to the p62 promoter is associated with lower p62 expression

Because the major risk factor for AD is age and p62^{-/-} mice possess an AD-like phenotype [20], to better understand the relationship between loss of p62 and AD, studies were undertaken to examine whether there are disturbances in p62 expression in AD. Postmortem human brain samples from middle and late stage AD individuals (Braak IV and VI, respectively) and controls with no neurological disease were employed (Table 1). Western blot analysis of lysates employing p62 monoclonal antibody revealed significantly higher p62 expression levels in human normal brain than samples obtained from AD brain (Fig. 4A). No alterations in actin levels were observed. DNA oxidative damage in three amplicons of the same five normal and seven AD samples were also examined. The average degree of damage exhibited by either

amplicon 1, 2 or 3 of the AD samples was significantly higher than that of normal samples (Fig. 4B). The correlation between the average degree of oxidative damage to the promoter and the relative expression of p62 these samples was analyzed. A significant negative correlation existed between the p62 expression level and the average oxidative damage to the 3 amplicons in the p62 promoter was identified ($r = -0.6765$, $p < 0.05$, Fig. 4C). Thus, high DNA oxidative damage contributes to low levels of p62 expression. To further define the relationship between p62 expression and promoter damage, human embryonic kidney cells (HEK) cells were treated with $H_2O_2/FeCl_2$ as previously described [19]. Treatment with increasing dose of H_2O_2 resulted in reduced p62 expression (Fig. 5A) along with concomitant increase in damage to all three amplicons in the p62 promoter (Fig. 5B). To extend this analysis to a relevant AD mouse model, the triple transgenic mouse model of AD (3xTg-AD), which exhibits many features of AD neuropathology [32], were employed. The 3xTg-AD mice harbor APP_{Swe}, Tau_{p301L}, and PS1_{M146V} human genes. NonTg mice were used from the same strain and they process the endogenous wild-type mouse PS1 gene. The expression of p62 and potential damage to the p62 promoter in these mice was examined. Relative p62 expression in NonTg mice was significantly higher than that in 3xTg-AD mice (Fig. 6A); however there were no alterations in the actin levels. The same samples were analyzed for p62 promoter damage; oxidative damage to the p62 promoter in 3xTg-AD mice was significantly higher than in NonTg mice (Fig. 6B). Altogether, the findings in the AD mouse model are congruent with the findings in human AD brain samples, further suggesting a functional relevance.

Discussion

Brain aging is associated with a progressive imbalance between intracellular levels of ROS and antioxidant defenses. Clearly, multiple cellular interactions are involved simultaneously in directing the aging process. Aberrant signaling and oxidative stress associated with aging have been reviewed recently and include age-related derangement of redox-regulated signals, age-related changes in anti-oxidant enzymes, age-related decrease in plasma cysteine, and dysregulation of iron and calcium homeostasis etc. [21]. Extensive studies reveal that DNA damage accumulates with age. For example, age-associated increases in tissue oxidative DNA damage in Sprague-Dawley rats correlated with 8-OHdG levels [33]. Also, in a type 2 diabetes model, 8-OHdG levels are increased in an age-dependent manner [27]. Our findings are in keeping with age as a factor for accumulation of oxidative damage. This is likely due to removal of DNA adducts by an anti-oxidant repair system efficiently in young and mature animals, but the production of ROS increases due to reduced antioxidant response and a decline in the capacity of DNA repair with age along with deficiency of p62.

Accumulating evidence demonstrates that p62 plays a yet to be fully understood role in neurodegenerative disease. For instance, p62 may protect neuronal cells from toxicity of misfolded proteins by enhancing aggregate formation under stress conditions [34], and aggresomes containing p62 have been observed in surviving cells [18]. Because of the link between transcriptional expression of p62 [18,35], and the GC content within the p62 promoter, as well as multiple transcription factor binding sites responding to diverse signals [30], we hypothesized a relationship between oxidative damage to the p62 promoter and age within this region. Damage to the mouse p62 promoter in mice increased with age, and damage to the human p62 promoter was also significantly age-correlated, revealing that age-related oxidative stress results in oxidative damage to p62 promoter. Moreover, loss of p62 enhanced oxidative stress and DNA damage. Higher 8-OHdG levels and oxidative damage were observed in p62^{-/-} mice compared to WT mice. It was recently reported that higher ROS production in p62-deficient cells is a consequence of impaired NF- κ B activation, and ROS production can further increase c-Jun N-terminal kinase (JNK) activation, which can lead to cell death [36]. Although the question of whether elevated DNA damage is a causative factor of aging or is only correlative with aging still can not be answered by present observations [37], parallel

observations in mouse models of sporadic and genetic forms of AD reveal that decreased p62 expression may enhance the appearance of AD markers.

The promoters of some down-regulated genes with age are oxidatively damaged and the base-excision repair enzyme, human OGG1 can recover the oxidative DNA damage [22]. Moreover, nuclear OGG1 levels and activity in mild cognitive impairment (MCI) and AD brains were decreased compared to normal subjects [38]. Prolyl isomerase Pin1 catalyzes the conversion between the *cis* and *trans* conformations of phosphorylated Ser/Thr-Pro motifs in peptides and involved in AD [39,40]. Reduced Pin1 levels associated with Pin1 promoter polymorphisms and oxidative inhibition of Pin1 were found in late-onset AD [41,42]. Oxidative damage to three amplicons in the human p62 promoter and the amplicon in the mouse p62 5'-flanking region were used as the index of p62 promoter damage. Age-associated oxidative damage to the p62 promoter was significantly correlated with p62 expression AD brains, human cells treated with H₂O₂ as well as the 3XTg-AD mouse model (Figs. 4,5,6). Samples with higher p62 expression have lower promoter damage, and samples with lower expression have higher promoter damage. In the human p62 promoter, transcriptional factor binding sites for PEA3 and Sp-1 are located within the amplicon 3, a site for Pu.1 is located in the amplicon 1, and amplicon 2 includes AP-1/TRE elements [30]. Oxidative modifications of guanine within AP-1 and Sp-1 binding sites have been shown to completely inhibit the binding of AP-1 and Sp-1 transcription factors to DNA [43]. Therefore, oxidative modification of transcription factor binding sites within the p62 promoter could account for down-regulated p62 expression. Thus, p62 is also an age-related promoter that is sensitive to oxidative modification resulting in decreased expression [22]. Further studies will be needed to pinpoint the transcription factor binding site involved in regulating p62 expression.

An absence of p62 leads to reduced nuclear localization of Nrf2, this study and another [28], revealing that p62 regulates Nrf2 activity by a yet-to-be-defined mechanism. The transcriptional activation of the phase II detoxification enzymes is linked to a *cis*-acting element called the antioxidant response element (ARE) which regulates constitutive and inducible gene expression. Nrf2 plays a central role in gene expression of phase II detoxification enzymes and some antioxidant genes [44]. In postmortem brain from AD individuals, Nrf2 levels in the nucleus are also decreased [29]. Interestingly, there are several links between p62 and AD: 1) p62 deficient mice possess AD-tau brain pathology along with learning and memory deficits, and neurodegeneration [20]; 2) p62 deficient brain exhibit a loss of Nrf2 nuclear localization [28], similar to AD brain [29]; and, 3) AD brain exhibits reduced levels of p62.

In summary, we have observed that age-associated oxidative damage to the p62 promoter is negatively correlated with p62 expression and reduced Nrf 2 activity (Fig. 7). Previously we have shown that loss of p62 results in accumulation of K63 polyubiquitin chains [45], and neurodegeneration [20]. Based on these results, we propose that age-associated oxidative damage to the p62 promoter leads to down-regulated p62 expression in AD brain, and thus contributes to enhanced oxidative stress through a feedforward mechanism. P62 is well studied as a scaffold for signaling pathways involved in neurotrophin signaling, ubiquitination, and inflammation [17]. At its C-terminal tail p62 possesses an ubiquitin associated domain. A major hallmark in AD and other neurodegenerative diseases is the accumulation of ubiquitin rich aggregates [46,47]. Soluble protofibrils, monomers, and small oligomers are cytotoxic [48]. The role of p62 is to capture these misfolded proteins and mediate their degradation by the proteasome, or autophagy when the proteasome is overwhelmed [49,50]. Therefore, a decline in p62 expression as a result of oxidative damage to the promoter would have functional consequences that impair degradation of aggregated proteins such as tau and others (Fig. 7). Altogether these findings suggest that decline in p62 expression may put one at risk for AD, and other diseases related to protein conformation and folding. In this context, increasing p62 levels might be a viable strategy to prevent a wide-spectrum of oxidative stress-related diseases.

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B **Fig. 1.**

Oxidative stress increased in an age-dependent manner. **(A)** Immunostaining of 8-OHdG (N45.1) in brains of WT and p62^{-/-} mice with age. After proteinase K treatment, 2, 6, 12 months WT and p62^{-/-} mice brain sections were stained with anti-8-OHdG monoclonal antibody (7.5 µg/ml) and processed with the MOM kit. Negative controls with no primary antibody controls are also shown. The results are representative of staining conducted on sections from 4 individual mice for each genotype. For each age, images were processed at 5 X, 10 X, and 40 X magnification. **(B)** Three corresponding regions from CA1, CA3, and dentate gyrus (DG) of the hippocampus from each section were scanned. The quantification of 8-OHdG for each section was expressed by the mean average pixel (Pixel total/region size). The relative intensities were averaged and reported as Mean $\pm$ S.D. Two-way ANOVA results were: Age, $F = 46.50, p \ll 0.05$; WT/p62^{-/-}, $F = 38.53, p \ll 0.05$; interaction = NS). Asterisk (*) indicates significant (< 0.05) multiple comparison adjusted p values for individual comparisons of WT vs p62^{-/-} data for each age class.

A **B** **Fig. 2.**

p62 mediates protection from oxidative stress *in vitro*. **(A)** WT and p62^{-/-} MEF cells were seeded in 96-well plates at 10,000 cells per well. After 48 h incubation, the cells were treated with various concentrations of H₂O₂ for 18 h. Cell viability was assessed using the CellTiter-Glo assay kit. The percentage of surviving cells is expressed as Mean $\pm$ S.E.M (N = 4). Statistical significance of WT compared to ^{-/-} values identified by asterisk, indicating multiple comparison adjusted $p < 0.05$. **(B)** Deficiency of the p62 prevents the Nrf2 translocation from cytosol to nuclei upon oxidative stress. WT and p62^{-/-} MEF cells were pre-treated with leptomycin B (20 ng/ml) for 8h followed by treatment with or without 500 μM or 2 mM

H₂O₂ for 30 minutes. Equal concentration of cytosolic protein (80 µg) was Western blotted with anti-Nrf2 and anti-tubulin antibodies as shown.

A **B**

C

Age	17	31	35	39	41	44	48	55	60	65	68	69	75	85	86	
Damage	28.2	25.1	23.7	17.3	28.5	31.9	27.5	36.4	38	27.4	25.8	42.8	27.5	39.6	39	$X = 30.6 \pm 7.1$

D

Fig. 3.

Oxidative damage to the p62 promoter is age-dependent. **(A)** Diagram of amplicons designed within the mouse and human p62 promoter. The amplicon was designed at -1801 ~ -1777 of the 5'-flanking region of the mouse p62 gene. Three amplicons, amplicon 1 (-1085 ~ -954), amplicon 2 (-407 ~ -321) and amplicon 3 (-1608 ~ -1484), were designed within the human p62 promoter. **(B)** Oxidative damage to the mouse p62 promoter in WT mice with increasing age. DNA was isolated from 2, 6 and 12 month wild type brain tissue, and then subjected to fpg digestion and Q-PCR with specific primer for the amplicon within the mouse p62 promoter. The oxidative damage to the promoter is expressed as Mean $\pm$ S.D (ANOVA $F = 10.74$, $p < 0.05$) from Tukey's studentized range tests. **(C)** Fpg-treated and non-treated genomic DNA

was used as a template and specific primers were used for absolute quantitative real time PCR assay. Oxidative damage to amplicon 3 was examined in 15 human brain tissues from age 17 to 86. **(D)** Oxidative damage was tested in 3 amplicons within the p62 promoter of samples from normal human brains of with various ages and a correlation analysis was conducted between DNA damage in amplicon 3 and age. Correlation coefficients and significance levels are indicated (**C**, **D**).

A

Fig. 4. p62 expression level is reduced in AD brain and correlated with oxidative damage within the p62 promoter region. **(A)** p62 expression in control and AD brains. An equal concentration of tissue homogenate (50 μ g) from control and AD brain lysates were subjected to 10% PAGE followed by western blot with anti-p62 and anti-actin antibodies. Expression levels of p62 was determined by scan of the blot. The data are expressed as relative intensity (Mean $\pm$ S.D., $**p < 0.01$). **(B)** Oxidative DNA damage percentage in 3 amplicons within the human p62 promoter. Total genomic DNA extracted from normal and AD brain tissues were digested by Fpg, then subject to real-time PCR with three sets of specific primers for 3 individual amplicons (Table 2). The Mean $\pm$ S.D. damage is reported ($*p < 0.05$). **(C)** Correlation analysis between

oxidative damage within the p62 promoter region and relative p62 expression. Correlation coefficient and significance levels are indicated.

A **B** **Fig. 5.**

P62 is oxidatively damaged in H_2O_2 -treated HEK cells. **(A)** HEK cells were treated with 100 μM , 300 μM and 500 μM H_2O_2 along with 20 μM , 60 μM and 100 μM FeCl_2 for 12 h. Equal amount of total lysate (40 μg) from non-treated and H_2O_2 -treated cells was Western blotted with anti-p62 and anti-actin antibodies. P62 expression level was expressed by a ratio in the relative intensity of the p62 signal to the actin signal. **(B)** Profile the oxidative damage to the p62 promoter from H_2O_2 -treated and non-treated HEK cells. The percentage of oxidative damage to each of the 3 amplicons within the p62 promoter was measured by QRT-PCR and averaged across all three amplicons. The data is presented as Mean damage $\pm$ S.D (* $p < 0.05$).

A **B** **Fig. 6.**

Reduced p62 expression and increased damage to the p62promoter. **(A)** Equal amounts of tissue homogenate protein (50 μ g) from WT and transgenic AD mice were western blotted with anti-p62 and anti-actin antibodies. p62 expression levels in normal and AD brains were expressed as the ratio of the relative intensity of the p62 blot to actin blot (Mean $\pm$ S.D., * p < 0.05). **(B)** Oxidative damage in the amplicon within the mouse p62 promoter region of NonTg and 3xTg-AD mouse brains. NonTg and 3xTg-AD mice brain tissues were used to prepare genomic DNA. Real-time PCR was performed with specific primers for the amplicon designed within the mouse p62 gene 5'-flanking region. * p < 0.05, significant difference.

Fig. 7.

Model illustrating the relationship between oxidative damage to the p62 promoter and p62 expression. Accumulating oxidative stress with age leads to the oxidative damage to the p62 promoter, resulting in reduced p62 expression. Decline in p62 levels would result in reduced delivery of substrates to the autophagosome, decreased Nrf2 nuclear translocation, leading to an increased level of protein aggregates, ROS, and misfolded substrates such as tau protein. Decline in p62 promotes a feedforward mechanism to accelerate oxidative damage and reduce phase II detoxification enzymes and antioxidant gene expression. Altogether, decline in p62 expression along with other factors could contribute to age-dependent neurodegenerative diseases such as AD and other neurodegenerative disease (ND).

Table 1

Control and AD brain samples

Brain Number	Distributive Dx	Age	Race/Sex	PMI	ApoE	Other disease	Source
OS00-06	Normal	60	b/f	8	E3/4		ADRC, Emory
OS00-23	Normal	68	w/f	11	E3/3		ADRC, Emory
OS01-112	Normal	65	w/f	6	E3/3		ADRC, Emory
OS02-35	Normal	75	w/f	6	E3/3		ADRC, Emory
OS03-299	Normal	69	w/m	2.5	E3/3		ADRC, Emory
OS00-05	Mild AD	74	w/m	<6	E2/3		ADRC, Emory
OS01-73	Mild AD	76	w/f	15	E3/3		ADRC, Emory
OS01-126	Mild AD	87	w/f	32	E3/4		ADRC, Emory
OS01-129	Mild AD	92	w/f	NA	E3/3		ADRC, Emory
OS00-38	Severe AD	92	w/f	6	E3/3		ADRC, Emory
OS01-02	Severe AD	69	w/f	5.5	E4/4		ADRC, Emory
OS01-11	Severe AD	80	b/f	5	E3/4		ADRC, Emory
BRC 747	Normal	86				-	ADRC, UAB
BRC 576	Normal	85				Aneurysm	ADRC, UAB
BRC 663	Normal	59				Breast Cancer	ADRC, UAB
BRC 511	Normal	59				Adenosarcoma	ADRC, UAB
BRC 399	Normal	39				Pulmonary Emboli	ADRC, UAB
5727	Normal	48	M	24.32	-		HBTRC
5813	Normal	41	M	27.17	-		HBTRC
5852	Normal	55	M	35.32	-		HBTRC
6951	Normal	17	M	28.92	-		HBTRC
5304	Normal	44	M	24.61	-		HBTRC
5352	Normal	31	M	32.92	-		HBTRC
5358	Normal	35	M	25.67	-		HBTRC

Table 2

Amplicons and Primer Pairs for Real-time PCR

P62 promoter	Amplicon	Primer sequence	Tm	
Human	Amplicon1	5'-CAT TCA CAC CTG TGG ACC AGC -3'	58.5	Sense
		5'-CTT GCA GGA GCT GGA GAA ACC-3'	58.3	Antisense
	Amplicon 2	5'-GAC CTA GCA GCC TCC TGA TAT GG-3'	58.7	Sense
		5'-TGG CCA TGA CTC AGC AAT ATC CTC-3'	58.8	Antisense
	Amplicon 3	5'-AGC TTC CCA AAG ACT CCC TCT TCT-3'	59.6	Sense
		5'-TCC CAT GAC TTT GAC TCA GCA GGT-3'	60.3	Antisense
Mouse	Amplicon	5'-TTT TCT CGC CAT TTG GCC CGT T-3'	60.2	Sense
		5'-ATG AGC TCT TAG CAG GAA CCC AGT-3'	59.8	Antisense